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Molecular mechanism of endothelial nitric-oxide synthase activation by *Platycodon grandiflorum* root-derived saponins

Hyung Gyun Kim[a,1], Tran Thi Hien[b,1], Eun Hee Han[a], Young Chul Chung[c], Hye Gwang Jeong[a,*]

[a] Department of Toxicology, College of Pharmacy, Chungnam National University, 220 Gung-dong, Yuseong-Gu, Daejeon 305-764, Republic of Korea
[b] Department of Pharmacy, College of Pharmacy, Chosun University, Gwangju, Republic of Korea
[c] Division of Food Science, Korea International University, Jinju, Republic of Korea

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ABSTRACT

Nitric oxide (NO) produced by endothelial nitric-oxide synthase (eNOS) has antithrombotic and antiatherosclerotic properties in the vasculature. Previously, we demonstrated that saponins derived from the roots of *Platycodon grandiflorum* (CKS) inhibited the tumor necrosis factor-α-induced expression of adhesion molecules in human endothelial cells. In this study, we found that CKS increased eNOS phosphorylation and NO production in human endothelial cells. Treatment with CKS increased the phosphorylation of Akt, p38/MAPK, AMP-activated protein kinase (AMPK), and calmodulin-dependent protein kinase II (CaMK II) leading to increased NO production in human endothelial cells. Moreover, inhibitors of Akt (LY294002), p38/MAPK (SB203580), AMPK (compound C), and CaMK II (W7) failed to suppress CKS-induced eNOS phosphorylation. In addition, CKS-induced eNOS phosphorylation was inhibited by the overexpression of a dominant-negative mutant form of AMPK (DN-AMPK). Taken together, these results indicate that CKS stimulates eNOS phosphorylation and NO production via the activation of PI3K/Akt, p38/MAPK, AMPK, and CaMK II.

1. Introduction

Endothelial dysfunction plays a pivotal role in the development of atherosclerosis by decreasing the bioavailability of endothelial nitric oxide (NO) (Nathan and Xie, 1994; Sessa, 2005). NO production, which is used as a marker of endothelial function, is mediated by endothelial nitric-oxide synthase (eNOS) upon the conversion of L-arginine to L-citrulline and plays a protective physiological role in the vasculature (Li and Förstermann, 2000).

The PI3K/Akt pathway is a crucial regulator of cellular proliferation, cell-cycle progression, and cell survival (Vivanco and Sawyers, 2002). Akt kinase is an important determinant of eNOS phosphorylation at Ser-1177, implying intimate involvement in the basal activation of eNOS and agonist-mediated stimulation (Bauer et al., 2003). Moreover, activation of the PI3K/Akt pathway leads to the phosphorylation of eNOS and increased NO production (Hashimoto et al., 2006).

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, including gene expression, mitosis, differentiation, and cell survival (Pearson et al., 2001). MAPKs also play important roles in sepsis and ischemic injury (Hsu et al., 2007), and are critical components of cardioprotection during ischemia and reperfusion (Jaswal et al., 2007). Moreover, Anter et al. (2004, 2005) found that eNOS activation is mediated by p38/MAPK, and that p38/MAPK activation prior to ischemia resulted in cardioprotection (Nakano et al., 2000). Thus, p38/MAPK regulates eNOS expression in cases of atherosclerosis and ischemic, sepsis-induced myocardial injury.

AMP-activated protein kinase (AMPK) was reported to phosphorylate eNOS at Ser-1177, which plays an important regulatory role in eNOS activity (Chen et al., 1999), and AMPK-induced phosphorylation was shown to induce NO production in endothelial cells (Morrow et al., 2003). In addition, calmodulin-dependent protein kinase II (CaMK II), an upstream modulator of the AMPK pathway, was identified as an AMPK kinase (Hurley et al., 2005). It has been shown that an increased intracellular free Ca^{2+}/calmodulin concentration ($[Ca^{2+}]$) affects eNOS. Ca^{2+}-mediated eNOS activation requires the ubiquitous Ca^{2+}-binding protein calmodulin (Busse and Mulsch, 1990), and eNOS expression is mediated through CaMK II in endothelial cells (David-Dufilho et al., 2005; Mount et al., 2008). Furthermore, the Ca^{2+}-ATPase inhibitor thapsigargin induces an increase in the $[Ca^{2+}]$ leading to constitutive NO synthase activation and NO production (Moritoki et al., 1994), and NO synthesis is dependent on CaMK II (Schneider et al., 2003). Thus, CaMK II is involved in NO synthesis and eNOS phosphorylation as a result of Ca^{2+}-dependent eNOS activation.

* Corresponding author. Tel.: +82 42 821 5936; fax: +82 42 825 4946.
E-mail address: hgjeong@cnu.ac.kr (H.G. Jeong).
[1] These authors contributed equally.

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Platycodi radix, the root of *Platycodon grandiflorum* A.DC. (Campanulaceae family), has been used as food and in traditional oriental medicine to treat bronchitis, asthma, pulmonary tuberculosis, and inflammation, as well as being used as a sedative (for a review of its biological significance, see Lee, 1973; Kim et al., 1995). Previous studies found that Changkil (CK), an aqueous extract of the roots of 20-year-old *P. grandiflorum* plants, prevented hypercholesterolemia and hyperlipidemia (Kim et al., 1995). Recently, it was shown that CK has protective effects against acetaminophen- and carbon tetrachloride-induced hepatotoxicity and inhibits the progress of hepatic fibrosis in rats (Lee et al., 2001, 2004; Lee and Jeong, 2002). In addition, a saponin fraction (CKS) has protective effects against acetaminophen- and carbon tetrachloride-induced hepatotoxicity (Lee et al., 2001; Lee and Jeong, 2002), and, inhibits both anaphylactic reactions and IgE-mediated allergic responses in mast cells (Han et al., 2009) and the TNF-α-induced expression of adhesion molecules in human endothelial ECV 304 cells (Kim et al., 2006).

Nonetheless, the effects of CKS on eNOS expression have not been characterized. In this study, we investigated whether CKS stimulates eNOS phosphorylation via PI3K/Akt, p38/MAPK, and AMPK through the up-regulation of CaMK II.

2. Materials and methods

2.1. Chemicals and materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY). LY294002, PD98059, SB203580, SP600125, W7 (N-[6-aminohexyl]-5-chloro-1-napthalenesulfonamide hydrochloride), compound C, DAF-2 DA (4,5-diaminofluorescein diacetate), DAF-2, and L-NAME were purchased from Calbiochem (La Jolla, CA). Antibodies against eNOS, phospho-eNOS, phospho-Akt, Akt, phospho-p38/MAPK, p38/MAPK, phospho-ERK, ERK, phospho-JNK, JNK, phospho-AMPK, AMPK, phospho-ACC, ACC, and phospho-CaMK II, as well as horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The tetrazole 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corp. (Cleveland, OH). The cytotoxicity detection kit used to measure lactate dehydrogenase (LDH) release was purchased from Roche Applied Science (Indianapolis, IN). All other chemicals were of the highest commercial grade available.

2.2. Preparation of CKS

CK refers to an aqueous extract obtained from 22-year-old roots of *P. grandiflorum*, which was supplied by Jang Saeng Doraji Co., Jinju, South Korea. The composition of *P. grandiflorum* root has been reported elsewhere (Kim et al., 1995). CK and CKS were prepared using a previously described method (Lee et al., 2001, 2004; Lee and Jeong, 2002). CK was subjected to column chromatography over amberlite XAD-2, Diaion MCl Gel HP20, or Kogel BG4600. After removal of the saccharides and amino acids with water, the column was eluted with methanol to obtain CKS as described previously (Tada et al., 1975).

2.3. Cell culture

ECV 304 cells were obtained from the American type culture collection (Bethesda, MD). The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and cultured in DMEM supplemented with 10% FBS. CKS was dissolved in dimethylsulfoxide (DMSO); stock solutions were added directly to the culture media. Control cells were treated with DMSO alone. The final solvent concentration was <0.1%.

2.4. Cytotoxicity of CKS in endothelial cells

Cells were plated in 48-well plates and cell viability was determined using conventional MTT reduction and LDH assays. After 24 h, various concentrations of CKS were added to each well and the plates were incubated at 37 °C for 24 h. Next, the supernatant was assayed for LDH at 490 nm using a microplate reader (Varioskan, Thermo Electron, Waltham, MA). The cells were treated with MTT solution for 1 h. The dark blue formazan crystals that formed in the intact cells were then solubilized with DMSO, and the absorbance was measured at 570 nm using a microplate reader (Varioskan, Thermo Electron). Cell viability (%) was calculated based on the absorbance relative to the absorbance of cells exposed to the control vehicle.

2.5. Western blot analysis

After treatment, the cells were collected and washed with phosphate-buffered saline (PBS) then lysed on ice for 30 min in 100 μl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), and 0.1% NP40 (Nonidet P-40)] and centrifuged at 12,000 rpm for 30 min. The supernatants were then collected, and the protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Aliquots of the lysates (40 μg of protein) were boiled for 5 min then subjected to 10% SDS-PAGE. The proteins in the gels were then transferred to nitrocellulose membranes, which were incubated with primary antibodies or mouse monoclonal anti-β-actin antibodies. The membranes were then further incubated with secondary anti-mouse or -rabbit antibodies. Finally, the protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce).

2.6. Measurement of NO production

NO production was assessed using the NO-specific fluorescent dye DAF-2 or DAF-2 DA (Calbiochem) as described previously (Leikert et al., 2001; Formoso et al., 2006). Briefly, ECV 304 cells were grown to 95% confluence in chamber slides (Lab-Tek, Rochester, NY) and serum-starved overnight. DAF-2 is membrane-impermeable. DAF-2 DA is a cell permeable compound that is converted to DAF-2 by intracellular esterases and forms a triazole derivative in the presence of NO that emits light at 515 nm upon excitation at 495 nm in proportion to the amount of NO present. DAF-2 DA was used in a number of studies detecting intracellular NO mainly by fluorescence microscopy. The cells were then loaded with DAF-2 or DAF-2 DA (final concentration, 1 μM) for 30 min at 37 °C, rinsed three times with DMEM, and kept in the dark. The cells were then treated with or without CKS as indicated in the figure legends. In some experiments, L-NAME (100 μM), LY294002 (10 μM), or SB203580 (10 μM) was added 30 min before loading with DAF-2 DA. In some experiments, compound C (10 μM) or W7 (10 μM) was

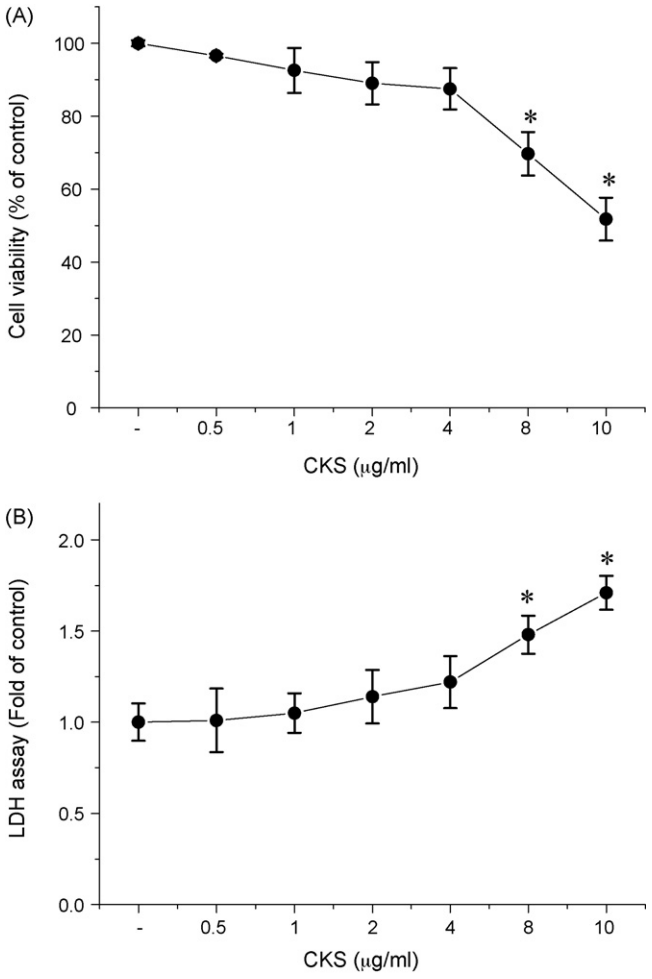

Fig. 1. Effect of CKS on cytotoxicity. Cells were plated in 48-well plates and exposed to various concentrations of CKS for 24 h. Cell viability was estimated based on (A) MTT and (B) LDH release assays. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from the control at $p < 0.05$.

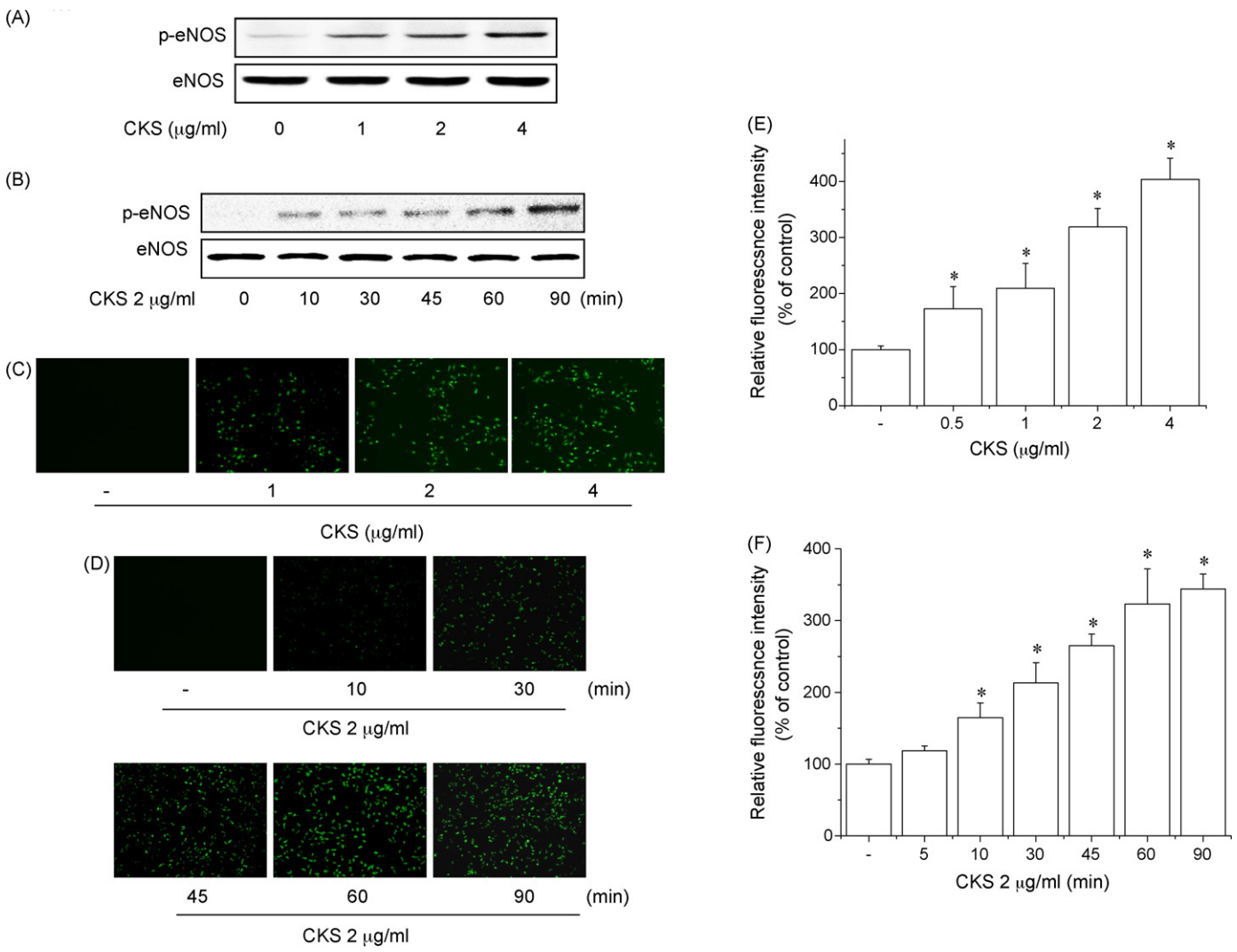

Fig. 2. Effect of CKS on eNOS phosphorylation and NO production. Cells were treated with 1–4 μg/ml of CKS for 1 h or 2 μg/ml of CKS for 10–90 min. The cells were then lysed, and the eNOS phosphorylation status at Ser-1177 was determined by immunoblotting with antibodies against phosphorylated or total eNOS. CKS increased eNOS phosphorylation in a dose-dependent manner. (A) CKS increased eNOS phosphorylation in a dose-dependent manner. (B) Under identical conditions, endothelial cells were treated with 2 μg/ml of CKS for 10–90 min. CKS increased eNOS phosphorylation following incubation for the indicated times. (C and D) The cells were serum-starved overnight then loaded with DAF-2 DA as described in Section 2. The cells were then stimulated with 1–4 μg/ml of CKS for 30 min or 2 μg/ml of CKS for the indicated times then fixed in 5% paraformaldehyde and visualized with an epifluorescence microscope as described in Section 2. The emission of green fluorescence is indicative of NO production. (E and F) NO production in response to CKS was detected with a peak excitation wavelength of 495 nm and a peak emission wavelength of 515 nm. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from the control at $p < 0.05$. (A–F) Representative results are shown for experiments that were repeated independently three times.

added 30 min before loading with DAF-2. The supernatants were then measured at 495/515 nm with a microplate reader (Varioskan, Thermo Electron). Finally, the cells were fixed in 5% paraformaldehyde for 5 min at 4 °C then visualized using an inverted epifluorescence microscope (Axiovert 200M; Carl Zeiss, Oberkochen, Germany) with an attached charge-coupled device camera using appropriate filters with a peak excitation wavelength of 495 nm and a peak emission wavelength of 515 nm.

2.7. Plasmid preparation and endothelial cell transfection

The dominant-negative AMPK plasmid (DN-AMPK) and control plasmid (pcDNA) were described previously (Lee et al., 2003). Cells were transfected with DN-AMPK or pcDNA in complete medium then cultured for 48 h prior to experimentation.

2.8. Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for p-values <0.05.

3. Results

3.1. Cytotoxicity of CKS in endothelial cells

Initially, we measured the cytotoxicity of CKS in endothelial cells using MTT and LDH assays. Dose-dependent cytotoxic effects of CKS were observed (Fig. 1A and B). CKS at the tested concentrations did not significantly affect the cytotoxicity with the exception of 8–10 μg/ml, which reduced the percent viability to 95%. Thus, the cells were exposed to CKS at a concentration of 0.5–4 μg/ml in our subsequent experiments.

3.2. CKS stimulates eNOS phosphorylation and NO production in endothelial cells

eNOS plays a protective physiological role in the vasculature (Li and Förstermann, 2000). Western blotting was performed to detect changes in the phosphorylation of eNOS in cells treated with 1–4 μg/ml of CKS or 2 μg/ml of CKS for 5–90 min. As shown

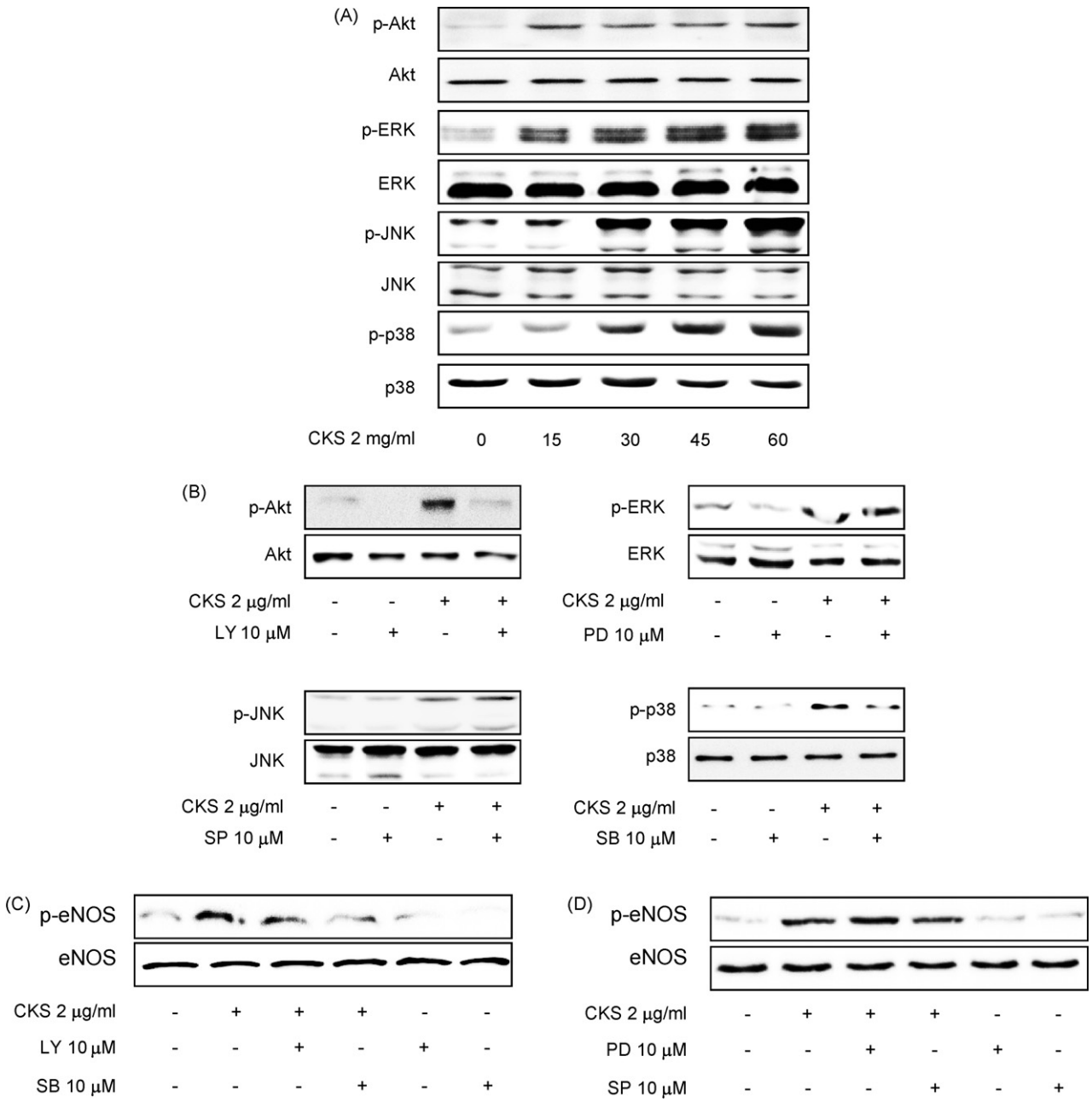

Fig. 3. Phosphorylation of eNOS by CKS via Akt and p38/MAPK phosphorylation. (A) Effect of CKS on the phosphorylation of Akt and MAPKs. Cells were treated with 2 μg/ml of CKS for the indicated times and then immunoblotted with activation-specific antibodies that recognize p-Akt, p-ERK1/2, p-p38/MAPK, and p-JNK1/2. The blots were then analyzed for the total kinase levels using anti-Akt, -ERK1/2, -p38/MAPK, and -JNK1/2 antibodies. (B) Effect of PI3K and MAPK inhibitors on the CKS-induced phosphorylation of Akt and MAPKs. Cells were preincubated with 10 μM LY294002, PD98059, SB203580, or SP600125 for 30 min and then incubated with 2 μg/ml of CKS for 30 min. Whole-cell lysates were subjected to Western blot analysis with p-Akt, p-ERK1/2, p-p38/MAPK, p-JNK1/2, or anti-Akt, -ERK1/2, -p38/MAPK, and -JNK1/2 antibodies. (C and D) Effect of PI3K and MAPK inhibitors on CKS-induced eNOS phosphorylation. Cells were preincubated with 10 μM LY294002, PD98059, SB203580, or SP600125 for 30 min and then incubated with 2 μg/ml of CKS for 60 min. Cell lysates were subjected to Western blot analysis for eNOS phosphorylation using anti-eNOS antibodies. (E) Under identical conditions, cells were serum-starved overnight then loaded with DAF-2 DA as described in Section 2. Cells were preincubated with the indicated inhibitors for 30 min and incubated with 2 μg/ml of CKS then fixed in 5% paraformaldehyde and visualized with an epifluorescence microscope as described in Section 2. The emission of green fluorescence is indicative of NO production. (F) NO production in response to CKS was detected with a peak excitation wavelength of 495 nm and a peak emission wavelength of 515 nm. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from the control at $p < 0.05$. **Significantly different from CKS-treated cells. (A–F) Representative results are shown for experiments that were repeated independently three times.

in Fig. 2A and B, eNOS phosphorylation was increased in a dose- and time-dependent manner by CKS. The quantity of protein in each lane was normalized to eNOS. To examine the stimulation of NO production by CKS, ECV 304 cells were loaded with DAF-2 DA, which fluoresces upon binding to an oxidized species of NO. As shown in Fig. 2C and D, green fluorescence (indicative of NO production) significantly increased in a dose- and time-dependent manner following 10–90 min of exposure to CKS. Furthermore, NO production was increased after treatment with CKS (Fig. 2E and F). These results indicate that CKS increases eNOS phosphorylation and NO production in endothelial cells.

3.3. CKS increases the phosphorylation of Akt and p38/MAPK in endothelial cells

To further elucidate the upstream signaling pathway involved in CKS-mediated eNOS phosphorylation and NO production, we

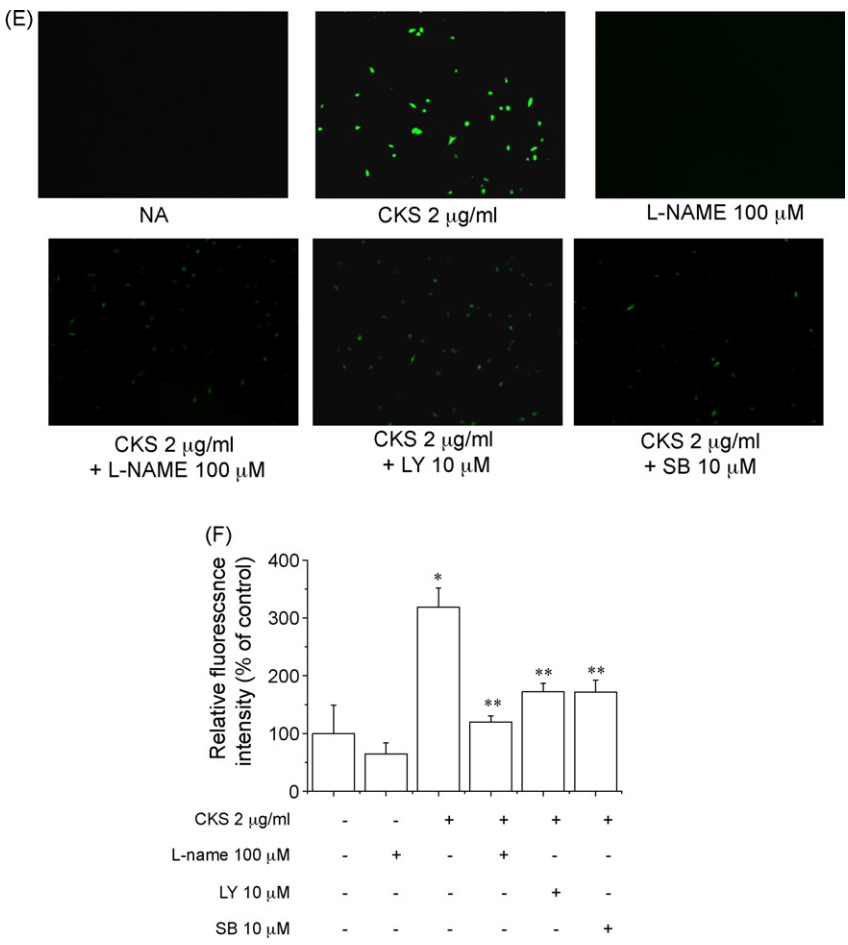

Fig. 3. (*Continued*).

examined the activation of PI3K/Akt, p38/MAPK, ERK1/2, and JNK1/2 in endothelial cells. As shown in Fig. 3A, the induction of Akt and phosphorylation of MAPKs were detected in CKS-treated endothelial cells by Western blotting using specific antibodies. No changes in the expression of total Akt or three MAPKs were detected, indicating that similar amounts of protein were loaded in each lane. To address the role of individual Akt and MAPK pathways in eNOS expression in response to CKS, cells were treated with LY294002, PD98059, SB203580, and SP600125, which are specific inhibitors of the PI3K/Akt, ERK, p38/MAPK, and JNK pathways (Fig. 3B), respectively. Whereas the inhibitors of ERK1/2 and JNK1/2 had no effect on CKS-induced eNOS phosphorylation, the inhibitors of the PI3K/Akt and p38/MAPK pathways significantly reduced CKS-induced eNOS phosphorylation (Fig. 3C and D). To test the stimulation of NO production by CKS via Akt and p38/MAPK signaling, green fluorescence was measured after treatment with CKS, L-NAME, LY294002, and SB203580. As shown in Fig. 3E, CKS-induced green fluorescence was reduced by LY294002 and SB203580. Moreover, CKS-induced NO production involved Akt and p38/MAPK signaling (Fig. 3F). Taken together, these data suggest that CKS increases eNOS phosphorylation in endothelial cells via Akt and p38/MAPK signaling.

3.4. Role of AMPK in CKS-induced eNOS phosphorylation

Previous studies have shown that the phosphorylation of eNOS at Ser-1177, which plays an important role in the regulation of eNOS activity, is induced by AMPK (Chen et al., 1999), and that AMPK-induced phosphorylation results in NO production in endothelial cells (Morrow et al., 2003). In the present study, we examined the effect of CKS on AMPK and ACC expression using cells treated with 2 μg/ml of CKS for 15–90 min. As shown in Fig. 4A, the phosphorylation of AMPK, ACC, and eNOS was increased by treatment with CKS for the indicated times. To test whether AMPK is necessary for eNOS phosphorylation, endothelial cells were treated with compound C, an AMPK inhibitor, prior to treatment with CKS. Our results indicate that the phosphorylation of AMPK and ACC was inhibited in endothelial cells (Fig. 4B) as compared with that in control cells. Treatment with compound C greatly attenuated the phosphorylation of eNOS, suggesting that AMPK is required for the phosphorylation of eNOS at Ser-1177. To examine whether AMPK activation is required for eNOS phosphorylation by CKS, endothelial cells were transfected with the control plasmid pcDNA or DN-AMPK prior to incubation with CKS for 24 h; marked attenuation of CKS-stimulated eNOS phosphorylation compared with the control plasmid-transfected cells was observed. eNOS phosphorylation was decreased by CKS in cells transiently transfected with DN-AMPK compared with CKS treatment alone (Fig. 4C). These data indicate that CKS increases the phosphorylation of eNOS in endothelial cells via the AMPK pathway.

3.5. CKS increases eNOS phosphorylation via AMPK through CaMK II

To examine whether CKS could activate CaMK II, endothelial cells were treated with 2 μg/ml of CKS for the indicated times then examined for CaMK II phosphorylation by Western blotting. Fig. 4D shows the increasing phosphorylation of CaMK II by CKS.

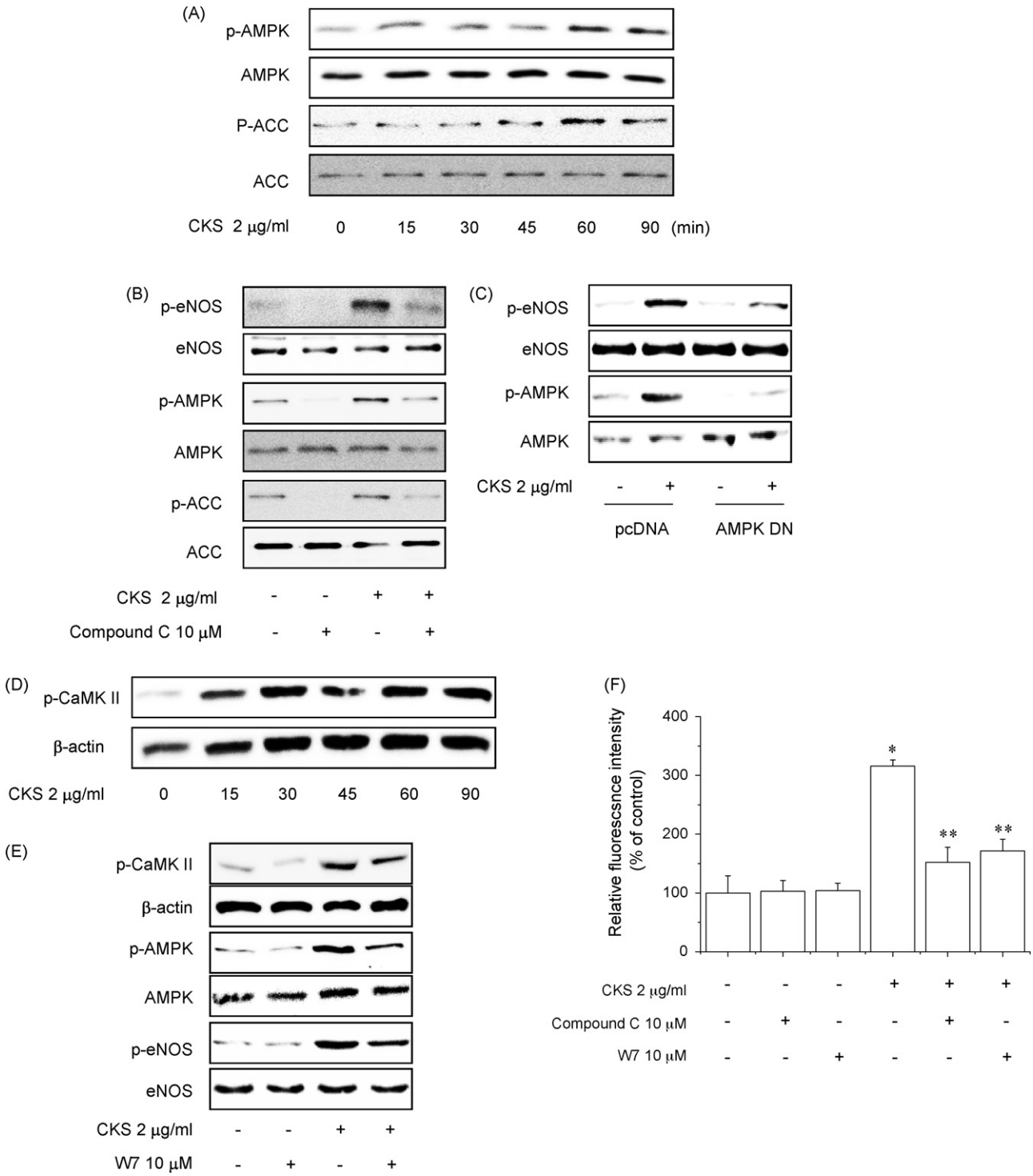

Fig. 4. Involvement of the AMPK pathway in CKS-induced eNOS phosphorylation through the up-regulation of CaMK II. (A) CKS-stimulated phosphorylation of AMPK and ACC in endothelial cells. (B) Cells were treated for 30 min with 10 μM compound C (an AMPK inhibitor) and then treated with 2 μg/ml of CKS for 60 min. Phosphorylated eNOS, AMPK, and ACC were detected by Western blot analysis. (C) Cells were transfected with DN-AMPK or pcDNA for 24 h then treated with 2 μg/ml of CKS for 60 min. Phosphorylated eNOS and AMPK were detected by Western blot analysis. (D) Western blot analysis of the CKS-increased phosphorylation of CaMK II. (E) Cells were treated for 30 min with 10 μM W7 (a CaMK II inhibitor) and then treated with 2 μg/ml of CKS for 60 min. Phosphorylated eNOS, AMPK, and CaMK II were detected by Western blotting. (F) Cells were preincubated with 10 μM compound C (an AMPK inhibitor) or W7 (a CaMK II inhibitor) for 30 min and then incubated with 2 μg/ml of CKS for 60 min. NO production in response to CKS was detected with a peak excitation wavelength of 495 nm and a peak emission wavelength of 515 nm. Each bar represents the mean ± SD calculated from three independent experiments. *Significantly different from the control at $p < 0.05$. **Significantly different from CKS-treated cells. (A–F) Representative results are shown for experiments that were repeated independently three times.

To investigate whether the CaMK II pathway is involved in the process by which CKS causes AMPK and eNOS phosphorylation, cells were treated with W7, a CaMK II inhibitor. As shown in Fig. 4E, W7 blocked CKS-induced eNOS and AMPK phosphorylation. Moreover, CKS-induced fluorescence was reduced by compound C and W7 (Fig. 4F). These data suggest a role for the CaMK II pathway in CKS-mediated eNOS phosphorylation through AMPK signaling.

4. Discussion

This is the first report to demonstrate that CKS activates eNOS phosphorylation via PI3K/Akt, p38/MAPK, and AMPK signaling. In addition, we found that CKS stimulates AMPK phosphorylation through CaMK II.

NO is a potent oxidant produced by both endothelial cells and macrophages that appears to exert both atherogenic and protective effects, depending on its source of production (De Caterina et al., 1995). NO production by the enzyme eNOS is one of the earliest events in atherogenesis (Knowles et al., 2000; Glass and Witztum, 2001). *P. grandiflorum* is commonly used in traditional oriental herbal medicine and has beneficial effects on inflammatory diseases (Lee, 1973). Previously, it was shown that CKS inhibits cytokine-induced cell adhesion molecule expression and reduces the adhesion of monocytes to endothelial cells (Kim et al., 2006). This study investigated the effects of CKS on the expression of eNOS in ECV 304 cells via two pathways. CKS increased PI3K/Akt/MAPK signaling and the phosphorylation of AMPK via CaMK II. These results suggest that CKS can prevent atherogenesis by increasing eNOS phosphorylation and inhibiting vascular inflammation. eNOS phosphorylation was stimulated by CKS in a time- and dose-dependent manner (Fig. 2A and B). In addition, CKS significantly increased the production of NO (green fluorescence) in endothelial cells in a time- and dose-dependent manner (Fig. 2C–F). The production and release of NO are important components of cardioprotection during ischemia and reperfusion (Boo et al., 2002; Niwano et al., 2003).

eNOS phosphorylation at Ser-1177 was first reported to be mediated by Akt (Dimmeler et al., 1999). Akt, a downstream target of PI3K, is the main kinase regulating eNOS phosphorylation and NO production under various conditions (Thomas et al., 2002; Cai et al., 2003). Our current experiments were designed to elucidate the possible role of the PI3K/Akt pathway in CKS-induced eNOS expression and NO production. CKS was found to activate PI3K/Akt. Furthermore, using LY294002, a specific inhibitor of PI3K/Akt, the CKS-stimulated phosphorylation of Akt and eNOS at Serine-1177 was markedly inhibited (Fig. 3B and C).

Apart from the fact that the PI3K/Akt pathway mediates eNOS phosphorylation, MAPK pathways have been reported to be involved in eNOS expression (Kan et al., 2008). Although it is generally believed that MAPKs do not play a crucial role in eNOS regulation, the present study was designed to determine whether MAPK pathways play a role in CKS-induced eNOS expression and NO production. CKS was found to activate the ERK1/2, P38/MAPK, and JNK1/2 pathways (Fig. 3A). In addition, using a specific inhibitor of the P38/MAPK pathway, we confirmed the involvement of p38/MAPK, but not ERK1/2 or JNK1/2, in CKS-induced eNOS expression (Fig. 3B and D). It was previously shown that p38/MAPK is activated in response to inflammatory cytokines, endotoxins, and osmotic stress (Pearson et al., 2001); however, its role in endothelial cells is incompletely defined. Our current data suggest that p38/MAPK is an upstream mediator of CKS-induced eNOS phosphorylation in endothelial cells.

The regulation of eNOS phosphorylation has also been characterized, and specific sites and protein kinases mediating the phosphorylation of eNOS have been reported. They include AMPK (Morrow et al., 2003), CaMK II (Wu, 2002), protein kinase A (PKA) (Boo et al., 2002), and protein kinase C (PKC) (Michell et al., 2001). In our study, CKS stimulated AMPK and ACC phosphorylation (Fig. 4A). To address the role of the AMPK pathway in the effects of CKS on eNOS phosphorylation using ECV 304 cells, we examined the effect of compound C (an AMPK inhibitor) on eNOS and AMPK phosphorylation. Moreover, the transfection of ECV 304 cells with AMPK-DN markedly attenuated CKS-stimulated AMPK and eNOS phosphorylation. In addition, an inhibitor of AMPK blocked the CKS-induced phosphorylation of eNOS (Fig. 4C).

Previous studies demonstrated that CaMKK acts as an upstream mediator of the AMPK pathway (Hurley et al., 2005) and regulates eNOS phosphorylation through the AMPK pathway (Mount et al., 2008). In our study, the phosphorylation of CaMK II was increased by CKS (Fig. 4D). Furthermore, W7 significantly inhibited CKS-induced eNOS and AMPK phosphorylation (Fig. 4E). Thus, CKS stimulates eNOS phosphorylation in ECV 304 cells via the AMPK pathway through the up-regulation of CaMK II.

In conclusion, CKS stimulates eNOS phosphorylation in ECV 304 cells via two pathways. First, CKS increases eNOS expression through Akt phosphorylation. Second, CKS stimulates eNOS phosphorylation via the AMPK pathway, which is dependent on the up-regulation of CaMK II.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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